Three-tier regulation of cell number plasticity by neurotrophins and Tolls in Drosophila

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Introduction

Balancing cell death and cell survival enables structural plasticity and homeostasis, regeneration, and repair and fails in cancer and neurodegeneration. In the nervous system, cell number plasticity is linked to neural circuit formation, adjusting neuronal number to functional requirements (Levi-Montalcini, 1987). In mammals, the neurotrophin (NT) protein family—NGF, brain-derived neurotrophic factor (BDNF), NT3, and NT4—regulates neuronal number through two mechanisms. First, full-length pro-NTs, comprised of a disordered prodomain and a cystine-knot (CK) domain, induce cell death; in contrast, mature NTs formed of CK dimers promote cell survival (Lu et al., 2005). Second, pro-NTs bind p75NTR and Sortilin receptors, inducing apoptosis via JNK signaling, whereas mature NTs bind p75NTR, promoting cell survival via NF-κB (Carter et al., 1996) and TrkA, B, and C, promoting cell survival via PI3K/AKT and MAPK/ERK (extracellular signal-related kinase; Lu et al., 2005). As the NTs also regulate connectivity and synaptic transmission, they couple the regulation of cell number to neural circuitry and function, enabling structural brain plasticity (Lu et al., 2005; Minichelli, 2009; Park and Poo, 2013). There is abundant evidence that cell number plasticity occurs in Drosophila melanogaster central nervous system (CNS) development, with neurotrophic factors including NTs and mesencephalic astrocyte-derived neurotrophic factor (MANF; Zhu et al., 2008; Palgi et al., 2009), but fruit flies lack p75NTR and Trk receptors, raising the question of how this is achieved in the fly. Finding this out is important, as it could lead to novel mechanisms of structural plasticity for both flies and humans.

The Drosophila NTs (DNTs) Spätzle (Spz), DNT1, and DNT2 share with mammalian NTs the characteristic structure of a prodomain and a conserved CK of 13–15 kD, which forms a disulphide-linked dimer (Hoffmann et al., 2008a,b; Zhu et al., 2008; Arnott et al., 2010; Hepburn et al., 2014). Spz resembles NGF biochemically and structurally, and the binding of its Toll-1 receptor resembles that of NGF to p75NTR (DeLotto and DeLotto, 1998; Mizuguchi et al., 1998; Arnott et al., 2010; Lewis et al., 2013; Hepburn et al., 2014). DNT1 (also known as spz2) was discovered by homology to BDNF, and DNT2 (also known as spz3) as a parologue of spz2 and DNT1 (Park et al., 2001; Zhu et al., 2008). DNT1 and 2 promote neuronal survival, and DNT1 and 2, Spz, and Spz3 are required for connectivity and synaptogenesis (Zhu et al., 2008; Sutcliffe et al., 2013; Ballard et al., 2014). Spz, DNT1, and DNT2 are ligands for Toll-1, -7, and...
-6, respectively, which function as NT receptors and promote neuronal survival, circuit connectivity, and structural synaptic plasticity (Weber et al., 2003; Zhu et al., 2008; McIlroy et al., 2013; Sutcliffe et al., 2013; Ward et al., 2015; McLaughlin et al., 2016). Tolls belong to the Toll receptor superfamily, which underlies innate immunity (Imler and Zheng, 2004; Leulier and Lemaître, 2008). There are nine Toll paralogues in flies, of which only Toll-1, -5, -7, and -9 are involved in immunity (Tauszig et al., 2000; Leulier and Lemaître, 2008). Tolls are also involved in morphogenesis, cell competition, and epidermal repair (Halfon et al., 1995; Yagi et al., 2010; McIlroy et al., 2013; Ballard et al., 2014; Carvalho et al., 2014; Meyer et al., 2014; Paré et al., 2014; Ward et al., 2015). Whether DNTs and Tolls can balance cell number plasticity is unknown.

Like the p75NTR receptor, Toll-1 activates NF-κB (a potent neuronal prosurvival factor with evolutionarily conserved functions also in structural and synaptic plasticity) signaling downstream (Hoffmann and Reichhart, 2002; Mattson and Meffert, 2006; Gutierrez and Davies, 2011). Toll-1 signaling involves the downstream adaptor MyD88, which forms a complex with Tube and Pelle (Horng and Medzhitov, 2001; Tauszig-Delamasure et al., 2002; Gay and Gangloff, 2007). Activation of Toll-1 triggers the degradation of the NF-κB inhibitor Cactus, enabling the nuclear translocation of the NF-κB homologues Dorsal and Dorsal-related immunity factor (Dif), which function as transcription factors. Other Tolls have also been suggested to activate NF-κB (McIlroy et al., 2013; Meyer et al., 2014). However, only Toll-1 has been shown to bind MyD88 (Tauszig-Delamasure et al., 2002), raising the question of how the other Tolls signal in flies.

Whether Tolls regulate cell death is also obscure. Toll-1 activates JNK, causing apoptosis, but its expression can also be activated by JNK to induce nonapoptotic cell death (Liu et al., 2015; Wu et al., 2015a,b). Toll-2, -3, -8, and -9 can induce apoptosis via NF-κB and dSarm independently of MyD88 and JNK (Meyer et al., 2014). However, in the CNS, dSarm induces axonal degeneration, but there is no evidence that it can promote apoptosis in flies (Osterloh et al., 2012). In other animals, Sarm orthologues are inhibitors of Toll signaling and MyD88 (Carty et al., 2006; Yuan et al., 2010), but there is no evidence that dSarm is an inhibitor of MyD88 in Drosophila. Thus, whether or how Tolls may regulate apoptosis in flies is unclear.

In the mammalian brain, Toll-like receptors (TLRs) are expressed in neurons, where they regulate neurogenesis, apoptosis, and neurite growth and collapse in the absence of any insult (Okun et al., 2011). However, their neuronal functions have been little explored, and their endogenous ligands in neurons remain unknown.

Because Toll-1 and p75NTR share common downstream signaling pathways and p75NTR can activate NF-κB to promote cell survival and JNK to promote cell death, we asked in this study whether the DNTs and their Toll receptors could have dual roles controlling cell survival and death in the Drosophila CNS.

Results

Different processing for each DNT ligand

Using 3D structural modeling based on the crystal structure of Spz (Lewis et al., 2013), we compared the mature CK domains of DNTs with those of mammalian NTs. They all share the structurally conserved CK unique to the NT family and distinct from those of other growth factors, with the characteristic arrangement of antiparallel β sheets and disulfide bridges (Fig. 1, A–D). The overhanging wings are out of phase by 90° in Drosophila versus mammalian ligands, possibly reflecting interactions with different receptor types (Fig. 1, B–D). The receptor-binding interface of Spz is not evolutionarily conserved in DNT1 or 2, suggesting distinct receptor affinities (Fig. 1 E). Thus, Spz, DNT1, and DNT2 are NT ligands with distinctive features.

The prodomains have distinctive features too. The prodomains of Spz and DNT2 are disordered coils, whereas that of DNT1 has helices, suggesting a globular structure (Figs. 2 A and S1). The DNT1 prodomain is also twice as long as that of DNT2. The prodomain of Spz has an α-helix just upstream of the Easter cleavage site, which undergoes a conformational change upon cleavage, essential for the activation of Toll (Arnott et al., 2010). This sequence is not conserved in the prodomains of the mammalian NTs nor in DNT1 and 2 (Fig. 2 A). This suggests that the activation mechanism of Toll by Spz is unique and distinct from those of Toll-6 and -7 by DNT2 and 1, respectively.

Mammalian pro-NTs are cleaved intracellularly by furin proteases or extracellularly by serine proteases (e.g., BDNF; Fig. 2 B). Spz is only secreted full length and is cleaved extracellularly by the serine proteases Easter or Spz processing enzyme (Hoffmann and Reichhart, 2002). Furin sites were absent from the Spz prodomain, but several highly conserved sites were found in DNT1 and 2 (Fig. 2 B). In vivo overexpression of mature Spz-CK, DNT1-CK, and DNT2-CK is functional and rescues the respective mutant phenotypes (Ligoxygakis et al., 2002; Hu et al., 2004; Zhu et al., 2008; Sutcliffe et al., 2013). However, S2 cells transfected with DNT1–C–terminal domain (CTD) tagged with 3×HA (DNT1-CK-CTD-HA) and DNT2-CK-HA did not secrete mature DNTs to the S2 cell medium (Fig. 2 D, lanes 3 and 8). This either suggests that the prodomain is required for trafficking in S2 cells or that S2 cells do not behave like neurons do in vivo. S2 cells transfected with wild-type full-length (FL) DNT1-FL-HA did not secrete DNT1-FL either but instead secreted a product truncated at the R283 site (Fig. 2 D, lane 2), suggesting that cleavage occurs naturally at this site. In contrast, S2 cells expressing DNT2-FL-HA invariably secreted the mature CK form of 15 kD (Fig. 2 D, lane 7). To test whether the conserved furin sites were responsible for these cleavage profiles, we performed site-directed mutagenesis of the furin sequences in HA-tagged DNT1 and 2 (Fig. 2 C). DNT1 lacking the furin site at R499 still secreted a product cleaved at R283, but no secreted protein was detected when both R499 and R283 were mutated (Fig. 2 D, lanes 4 and 5). Thus, the DNT1 furin site at R283, which is the most conserved, is functional. Mutagenesis of the DNT2 furin site R284 resulted in the secretion of two products of 30 kD and 18 kD (Fig. 2 D, lane 10). The 30-kD product corresponds to cleavage at site R284 predominates. The 18-kD product was not detectable in the media expressing wild-type DNT2, suggesting that it does not occur naturally and is the result of nonfurin cleavage. Mutagenizing R214, R221, and R284 sites resulted in the secretion of DNT2-FL-HA from S2 cells, showing that DNT2 can be secreted full length (Fig. 2 D, lane 11). These findings showed that the DNT2 furin cleavage site at R284 is functional and is the predominant cleavage site.

To test whether similar DNT processing occurs in vivo, we overexpressed in the retina (with GMR-GAL4) full-length forms tagged at the C termini with GFP and visualized the re-
sulting products with anti-GFP in Western blots. DNT1 was predominantly found in full-length form and also cleaved at furin sites at 98 (less abundant), 283 (pro-DNT1), and 499 (DNT1-CK-CTD; Fig. 2 E). DNT2 was found full length, but predominantly in mature form (DNT2-CK; Fig. 2 E). These data show that in vivo, DNTs are cleaved by furins and can be found in both pro- and mature forms.

To conclude, each DNT has unique features. DNT1 is more likely found in pro-form than DNT2, and DNT2 is more likely found in mature form. Ultimately, the forms secreted in vivo will depend on the expression profile of proteases and will be context dependent. The distinct processing mechanisms of Spz, DNT1, and DNT2 suggest functional differences.

Pro-DNT1 activates proapoptotic and mature DNT prosurvival pathways

To ask whether different DNT forms could have distinct functions, we tested whether they could activate proapoptotic or prosurvival signaling pathways.

Overexpression of mature DNT1 and 2 promotes cell survival in embryos (Zhu et al., 2008). In mammals, apoptosis is activated by pro-NTs binding p75NTR and activating JNK (Roux and Barker, 2002). Thus, we asked whether the different DNT forms activate JNK signaling, visualized using antiphospho-JNK antibodies. Overexpression of DNT1-CK-CTD, DNT2-CK, or DNT2-FL in the retina reduced the number of pJNK+ cells compared with controls, whereas overexpression of DNT1-FL increased pJNK+ cell number (Fig. 3 A). Most likely, DNT2-FL was cleaved intracellularly and secreted as mature CK instead (Fig. 2, D and E). Thus, pro-DNT1 can activate the JNK proapoptotic signaling pathway.

We next tested whether DNTs can activate the prosurvival pathways NF-κB and ERK. Stimulating S2 cells with purified mature DNT2-CK induced the phosphorylation of Dorsal (i.e., activation; Fig. 3 B). We also transfected S2 cells with Toll-6 or -7, stimulated them with purified mature DNT2-CK, and tested whether it triggered the nuclear translocation of Dorsal or Dif, thus activating NF-κB signaling. Subcellular fractionation revealed that DNT2 induced the degradation of the NF-κB inhibitor Cactus in the cytoplasm and the nuclear translocation of both Dorsal and Dif (Figs. 3 C and S2 A). These data demonstrate that mature DNT2-CK activates NF-κB signaling. Stimulation with DNT2-CK also activated signaling in nontransfected control cells (Fig. 3 C). Because S2 cells express multiple Tolls, but not Toll-6 (Fig. S2 B), this means that DNT2 can also bind other Toll family receptors. In fact, DNT1 binds Toll-7 and DNT2 binds Toll-6 (McIlroy et al., 2013), but DNT1 could also bind Toll-6 and DNT2 could also bind Toll-7 (Fig. S3). Thus, binding of DNT1 and 2 to Toll-6 and -7 is promiscuous. Importantly, both Cactus degradation and nuclear translocation of Dorsal and Dif induced by DNT2 were more pronounced in transfected cells than in mock controls (Fig. 3 C). This shows that Toll-6 and -7 activate NF-κB signaling downstream of DNT2.

To test whether DNTs, Toll-6, and Toll-7 could activate ERK, we overexpressed them and visualized activated antiphospho-ERK. Overexpression of either DNT1-FL or mature spz-CK in neurons of the larval brain optic lobe (with yellow, and blue triangles indicate conserved areas. Of 33 Toll-contact residues in Spz, only 11 are conserved in DNT1 and 2, with a single identical residue Tyr64 in Spz.
Figure 2. DNT1 and 2 are cleaved by conserved furin proteases. (A) The prodomain α-helix of Spz (boxes) required to activate Toll-1 is not conserved in DNT1 and 2. Yellow highlights indicate corresponding sequences that are not conserved. (B) The prodomains of DNT1 and 2 but not Spz have conserved furin sites. c.s., cleavage site. (C) Site-directed mutagenesis of furin sequences. Red letters mark amino acid substitutions. (D) Mutant DNT1-FL-HA and DNT2-FL-HA forms expressed in S2 cells and visualized in Western blots with anti-HA from lysate and secreted medium. Black arrows indicate normal forms, and red arrows indicate mutant products. (E) Anti-GFP Western blot upon overexpression of C-terminally tagged DNTs in the retina with GMR-GAL4 shows that furin cleavage occurs in vivo (black arrows: DNT1, blue arrows: DNT2). Molecular masses on the left of each blot are given in kilodaltons.
Figure 3. **DNTs and Tolls activate proapoptotic and prosurvival pathways.** (A) Overexpression of DNTs in larval retina with GMR-GAL4–altered pJNK activation. The box plot graph depicts a one-way analysis of variance: ***, P < 0.001; Dunnett’s post-hoc test. (B) Stimulation of S2 cells with purified DNT2-CK–induced Dorsal phosphorylation. (C) Stimulation of S2 cells with DNT2-CK provoked the degradation of the cytoplasmic inhibitor Cactus (αCact) and the nuclear translocation of Dif (αDif) and Dorsal (αDI), particularly in Toll-6– or -7–transfected cells. Molecular masses are given in kilodaltons. Dotted lines indicate that intervening lanes have been spliced out. (D) Overexpression of DNT1-CK-CTD and DNT2-CK, but not DNT1-FL or spz-CK, activated ERK (arrows) in RG-GAL4 neurons of the larval optic lobe. n = 5–11. (E) Overexpression of activated Toll-6CY and -7CY in the retina increased pERK (arrows indicate morphogenetic furrow [mf]). GMR-GAL4>TOR is a positive control. Error bars display SD (s.d.). One-way analysis of variance: P < 0.0001; Dunnett’s post-hoc test. n = 8–13. A, anterior; P, posterior. (F) Distinct effect of loss and gain of function for Tolls in Eve+ neuron numbers in larvae. Dashed lines indicate the median (left graph) or 50% of the data distribution in controls (right graph). One-way analysis of variance: ***, P < 0.0001; Dunnett’s post-hoc test. n = 5–22. ns, not significant. Asterisks on graphs indicate post-hoc multiple comparisons corrections: **, P < 0.01; ***, P < 0.001. > indicates GAL4/UAS. Bars, 50 µm. (G) Different ligand forms and Toll receptors can induce either cell survival or death. For genotypes, statistical details, and sample sizes, see Table S2.
with a lethality phase at pupariation, indicating this is a critical time for MyD88 function. Using anti–Death Caspase 1 (Dcp1), we counted all dying cells throughout the VNC of white pupae using adapted DeadEasy Caspase software (Forero et al., 2009). In MyD88<sup>+/−</sup> homozygotes, apoptosis levels did not differ from controls, but they increased in MyD88<sup>cr2.8</sup> /Df(2R)BSC279 transheterozygotes (Fig. 4 E). We generated a MyD88-null allele using clustered regularly interspaced short palindromic repeats (CRISPR)/Cas9, MyD88<sup>cr2.8</sup>/Df(2R)BSC279 pupae also had increased apoptosis (Fig. 4 E). Thus, MyD88 is required for neuronal survival. Collectively, these data show that Toll-6 and -7 signal via the canonical MyD88 pathway to promote neuronal survival in the CNS.

However, overexpression of MyD88 in all neurons also increased apoptosis in pupae (Fig. 4 E). This could occur downstream of Tolls, as overexpression of activated Toll-6<sup>CY</sup> or Toll-1<sup>106</sup> also increased apoptosis in pupae (Fig. 4 E). Remarkably, the proapoptotic effect of Toll-6 was enhanced when overexpressed in a MyD88<sup>+/−</sup> mutant background (Fig. 4 E), suggesting that Toll-6 might induce apoptosis in pupae independently of MyD88.

These data raised two questions: How does MyD88 induce apoptosis? And how can Toll-6 induce apoptosis independently of MyD88?

**Toll-6 can induce apoptosis via the MyD88 inhibitor dsarm**

In mammals, Sarm1 inhibits MyD88 and can induce neuronal apoptosis (O’Neill and Bowie, 2007; Carlson et al., 2016). Thus, we wondered whether *Drosophila* dsarm might be involved in proapoptotic signaling by Toll-6. We overexpressed dsarm in all neurons using EP3610 flies, which drive expression of multiple Ect4 isoforms (Ect4 is a synonym of dsarm). Elav>EP3610 increased apoptosis in pupal VNCs (Fig. 5 A). Remarkably, overexpression of dsarm in a MyD88<sup>+/−</sup> mutant background increased apoptosis further (Fig. 5 A). This showed that dsarm promotes apoptosis and antagonizes MyD88 function. Apoptosis led to neuronal loss, as overexpression of dsarm in normal or MyD88 mutant pupae decreased Eve<sup>+</sup> neuron number (Fig. 5 B). Because sarm mutants are embryonically lethal, to further verify this, we looked at the embryonic CNS. dsarm is expressed throughout the embryonic CNS, as visualized with a dsarm<sup>MIMIC-GFP</sup> reporter (Fig. 5 C). Overexpressing dsarm using either EP3610 or a single dsarm isoform (Osterloh et al., 2012) in all embryonic CNS neurons caused Eve<sup>+</sup> neuron loss (Figs. 5 D and S4). Conversely, dsarm<sup>UAS</sup>/dsarm<sup>UAS</sup> mutant embryos had more Eve<sup>+</sup> neurons (Figs. 5 D and S4). Collectively, these data show that dsarm induces apoptosis and neuronal loss.

**JNK is a common proapoptotic effector activated by p75<sup>NTR</sup> and Sarm1 in mammals and Tolls in flies**

Roux and Barker, 2002; Kim et al., 2007; Wu et al., 2015a). Thus, to ask whether dsarm induces apoptosis by activating JNK, we tested whether JNK knockdown could rescue apoptosis caused by dsarm overexpression. Indeed, overexpressing dsarm in all neurons together with JNK-RNAi decreased apoptosis compared with Elav>EP3610 (Fig. 5 A). Thus, dsarm activates apoptosis via JNK. To further verify this, we asked whether MyD88 and dsarm affected activated pJNK<sup>+</sup> cells in larval retina. MyD88<sup>+/−</sup>/Df(2R)BSC279 mutants had normal pJNK<sup>+</sup> cell numbers, but overexpressing dsarm increased pJNK<sup>+</sup> cell numbers (Fig. 5 E), and this increased further in a MyD88<sup>+/−</sup> mu-
Figure 4. **Toll-6 promotes cell survival via MyD88.** (A) Coimmunoprecipitations showing that MyD88-V5 bound Toll-6–Flag and Toll-7–Flag and activated Toll-6CY–Flag and Toll-7CY–Flag. IB, immunoblot; IP, immunoprecipitation. Molecular masses are given in kilodaltons. (B) Anti-MyD88 and exon trap reporters Dorsal-GFP and Dif-GFP visualized with anti-GFP are distributed throughout the embryonic CNS neuropile. Left, horizontal views; right, transverse sections; white arrows indicate reporter distribution within the neuropile. (C) Loss of Eve + neurons (arrows) in the CNS in MyD88 kra56 Toll-7P8 Toll-626 triple mutant embryos. For quantification, see Fig. S4. aCC, anterior corner cell; EL, Eve lateral. (D) Altering MyD88 signaling affects Eve + neuron number. Dashed lines indicate 50% (left graph) or 100% (right graph) data distribution in controls. Box plots: larvae, one-way analysis of variance, P < 0.001, Dunnett's post-hoc test; pupae, Welch's analysis of variance, P < 0.01, Dunnett's post-hoc test. n = 8–12. (E) Apoptotic cells visualized with anti-Dcp1 in white pupal.
tant background (Fig. 5 E). This showed that dSarm activates apoptosis via JNK and antagonizes MyD88 function.

To test whether dSarm could inhibit MyD88 through direct physical interaction, we performed coinmunoprecipitations. S2 cells were cotransfected with MyD88 tagged with V5 and dsarm tagged with HA. Precipitating MyD88 copurified dSarm, showing that dSarm and MyD88 interact physically (Fig. 5 F). Altogether, our data show that Sarm is an inhibitor of MyD88 and it induces apoptosis by antagonizing MyD88 and by activating JNK signaling.

If neuronal apoptosis depends on dSarm, why did MyD88 induce apoptosis in pupae? We had shown that overexpression of MyD88 increased neuron overexpression, overexpression of cactus decreased Eve+ neuron number, and MyD88 loss of function did not affect pJNK cell number, implying that NF-κB does not directly promote apoptosis. Importantly, apoptosis caused by MyD88 overexpression in neurons was rescued by JNK-RNAi knockdown (Fig. 5 A), meaning that apoptosis downstream of MyD88 requires JNK. This suggests that MyD88 might induce apoptosis by up-regulating the expression of JNK, wek (wek), or dsarm.

Our data had shown that Toll-6 can induce apoptosis and that it functions upstream of MyD88 to maintain neuronal survival, but MyD88 is inhibited by Sarm, which also induces apoptosis via JNK. So we asked whether Toll-6 and -7 could activate apoptosis by directly interacting with dSarm by using coinmunoprecipitations. We cotransfected S2 cells with Toll-6–Flag or -7–Flag and dsarm–HA and found that precipitating Toll-6 or -7 did not coprecipitate dSarm (Fig. 5 F). Thus, dSarm does not bind Toll-6 or -7, meaning that dSarm does not directly mediate the proapoptotic function of Toll-6.

Thus, our data show that Toll-6 functions upstream of dSarm and MyD88 to regulate neuronal death and survival, respectively (Fig. 5 G). But these data raise further questions: How can Toll-6 induce apoptosis if it does not bind dSarm? And why does Toll-6 promote cell survival in embryos and apoptosis in pupae?

Adaptor profiles change in space and time

Our data suggest that the relative levels of MyD88, dSarm, and Wek could determine neuronal life or death. Thus, we used MyD88-GAL4 to ask how increasing the levels of Wek and Sarm relative to normal MyD88 levels would affect neurons. Overexpression of wek in MyD88+ cells decreased Eve+ neuron numbers in pupae compared with controls, and overexpression of dsarm (EP3610) decreased Eve+ neurons further (Fig. 7 A). Using the nuclear reporter Histone-YFP, overexpression of wek reduced cell numbers in pupae, and overexpression of dsarm reduced cell numbers even further (Fig. 7 B). Remarkably, concomitant neuronal overexpression of wek with MyD88 knockdown resulted in the most severe cell loss in pupal VNCs (Fig. 7 B). Because overexpression of wek alone had only a mild effect, this reveals that normally Wek is in a tug of war between dSarm and MyD88 signaling, that MyD88 and dSarm have antagonistic functions regulating cell numbers, and that Wek can engage both pathways downstream of Toll-6. Thus, relative levels of Wek, Sarm, and MyD88 determine cell survival or death downstream of Tolls.

Toll-6 maintains neuronal survival in embryos and can promote both neuronal survival and death in pupae, suggesting that its signaling adaptors change over time. To test this, we used real-time quantitative RT-PCR (qRT-PCR) and measured MyD88, dsarm, and wek transcript levels in whole stage 17 embryos and in the dissected CNS of second and third instar larvae (L2 and L3) and 1-d-old pupae. MyD88 mRNA levels were high in embryos, decreased in L2 CNS, and increased again between L3 and white pupae (Fig. 7 C). Relative to MyD88 transcripts, dsarm mRNA levels were high in embryos, decreasing thereafter (Fig. 7 C), and wek mRNA levels were virtually absent in embryos and increased from L2 on (Fig. 7 C). wek expression was consistently lower than that of dsarm and equal to MyD88 from L2 onward (Fig. 7 C). The low levels of Wek in embryos suggest that in the embryonic CNS, Toll-6 can bind MyD88 to activate cell survival, but because there is no Wek, it cannot activate the dSarm proapoptotic pathway. In the pupa,
Figure 5. **dSarm antagonizes MyD88 and promotes apoptosis downstream of Toll-6.** (A) Apoptotic cells visualized with anti-Dcp1 in pupal VNCs and quantified with DeadEasy. Box plots: left, Welch's analysis of variance, P < 0.0001, Bonferroni's post-hoc test; right, one-way analysis of variance, P < 0.0001, Tukey's post-hoc test. n = 9–16, ns, not significant. (B) Eve+ neuron numbers in the abdominal VNCs of L3 larvae are regulated by dSarm. Box plot: one-way analysis of variance, P < 0.0001, Dunnett's post-hoc test. n = 9–12. (C) Anti-GFP in dsarm\(^{4705-4921}\) is distributed throughout the embryonic CNS neuropile. (D) Loss and gain of dsarm function affects Eve+ neuron numbers in embryos (black arrows indicate neuronal loss, and red arrows indicate supernumerary neurons). For quantification, see Fig. S4. aCC, anterior corner cell; EL, Eve lateral. (E) dSarm can activate JNK signaling, seen with anti-pJNK in the larval retina. Box plot: one-way analysis of variance, P < 0.001, Dunnett's post-hoc test. n = 4–18. (F) Coimmunoprecipitation from S2 cells showing that dSarm binds MyD88, but does not bind Toll-6 or Toll-7. Arrows point to relevant bands. IB, immunoblot; IP, immunoprecipitation. Molecular masses are given in kilodaltons. (G) dSarm inhibits MyD88 and activates JNK, promoting apoptosis. Asterisks on graphs indicate post-hoc multiple comparisons corrections: **, P < 0.01; ***, P < 0.001. See Table S2. > indicates GAL4/UAS. Bars: (A, C, and E) 50 µm; (B) 100 µm.
in the presence of Wek, Toll-6 can activate either cell survival via MyD88 or cell death via dSarm. Thus, the temporal regulation of wek expression explains the different outcomes of Toll-6 function over time.

To visualize whether the spatial distribution of MyD88 and dSarm may also change, we used a dsarmMIM IC-GFP insertion and MyD88-GAL4 NP6394 to drive the expression of membrane-tethered 10xUAS-myr-td-Tomato and anti-DsRed antibodies. Both were widely expressed throughout the embryonic CNS neuropile (Figs. 4 and 5), widespread in larvae (Fig. 7 D), and more restricted in pupae (Fig. 7 E). In pupae, MyD88>myr-td-Tomato was distributed throughout the VNC, but prominently in thoracic interneurons potentially linked to the motor circuitry (Fig. 7E). dSarmMIM-GFP was distributed...
throughout the VNC but prominently in ventral projections, apparently sensory circuits (Fig. 7 E). These distinct patterns suggest that after cell number regulation, neural circuits acquire a characteristic composition of Toll signaling adaptors.

Mammalian NTs can induce signaling from mammalian TLRs

To test whether the link between NTs and Toll receptors might also occur in mammals, we performed signaling assays with TLR2 and TLR4, which are cell membrane receptors present in the mammalian brain, and TLR5, an intracellular receptor (Gay et al., 2014). HEK293T cells were transfected with TLR2, 4, and 5 and an NF-κB luciferase reporter, and signaling was measured after stimulation with increasing concentrations of mature BDNF or NGF (Fig. S5). Whereas there was no effect upon stimulation of TLR2 or TLR5 with either NGF or BDNF, both ligands induced signaling in cells transfected with TLR4 (Fig. S5). Furthermore, treatment with NGF or BDNF altered the response of TLR2, 4, and 5 to stimulation with their canonical innate immunity ligands (Fig. S5). This means that mammalian NTs can influence mammalian TLR signaling.
Discussion

DNTs and Tolls regulate cell number plasticity by promoting both cell survival and death in the Drosophila CNS through a three-tier mechanism.

In the first tier, each DNT has unique features conducive to distinctive functions (Fig. 8 A). Spz, DNT1, and DNT2 share with the mammalian NTs the unequivocal structure of the CK domain unique to this protein family. However, DNT1, DNT2, and Spz have distinct prodomain features and are processed differently, leading to distinct cellular outcomes (Fig. 8 B). Spz is only secreted full length and cleaved by serine proteases (Hoffmann and Reichhart, 2002). DNT1 and 2 are cleaved intracellularly by conserved furins. In cell culture, DNT1 was predominantly secreted with a truncated prodomain (pro-DNT1), whereas DNT2 was secreted mature. In vivo, both pro- and mature DNTs were produced from neurons. Interestingly, DNT1 also has an isoform lacking the CK domain (Zhu et al., 2008), and Spz has multiple isoforms with truncated prodomains (DeLotto et al., 2001). Thus, in vivo, whether DNT1 and 2 are secreted full length or cleaved and whether Spz is activated will depend on the proteases that each cell type may express. Pro-DNT1 activates apoptotic JNK signaling, whereas mature DNT1 and 2 activate the prosurvival NF-κB (Dorsal and Dif) and ERK signaling pathways. Mature Spz does not activate ERK. This first tier is evolutionarily conserved, as mammalian pro-NTs can promote cell death, whereas furin-cleaved mature NTs promote cell survival (Lu et al., 2005). NF-κB, JNK, and ERK are downstream targets shared with the mammalian NTs, downstream of p75NTR (NF-κB and JNK) and Trks (ERK), to regulate neuronal survival and death (Roux and Barker, 2002; Lu et al., 2005; Minichiello, 2009). Thus, whether a cell lives or dies will depend on the available proteases, the ligand type, and the ligand cleavage product it receives (Fig. 8 A).

In a second tier, we showed that the specific Toll family receptor activated by a DNT matters (Fig. 8 B). Toll-6 and -7 could maintain neuronal survival, whereas Toll-1 had a predominant proapoptotic effect. Because there are nine Tolls in Drosophila, some Tolls could have prosurvival functions, whereas others could have proapoptotic functions. Different Tolls also lead to different cellular outcomes in immunity and development (Tauszig et al., 2000; Yagi et al., 2010; Meyer et al., 2013; McIlroy et al., 2013; Paré et al., 2014). Thus, the life or death of a neuron will depend on the Toll or combination of Tolls it expresses (Fig. 8 B). We also showed that binding of Spz to Toll-1 is most likely unique, but DNT1 and 2 bind Toll-6 and -7 promiscuously, and, additionally, we showed that DNT1 and 2 with Toll-6 and -7 activate NF-κB and ERK, whereas pro-DNT1 activates JNK. This suggests that ligand prodomains might alter the affinity for Toll receptors and/or facilitate the formation of heterodimers between different Tolls and/or with other coreceptors to induce cell death. A “DNT–Toll code” may regulate neuronal numbers.

In a third tier, available downstream adaptors determine the outcome between cell survival and death (Fig. 8 C). Toll-6 and -7 activate cell survival by binding MyD88 and activating NF-κB and ERK (whether ERK activation depends on MyD88 is not known), and Toll-6 can activate cell death via Wek, dSarm, and JNK signaling. We have shown that Toll-6 binds MyD88 and Wek, which binds dSarm, and that dSarm binds MyD88 and promotes apoptosis by inhibiting MyD88 and activating JNK. Wek also binds MyD88 and Toll-1 (Chen et al., 2006). So, evidence suggests that Wek recruits MyD88 and dSarm downstream of Toll-6 (Fig. 8 C). Because Toll-6 binds both MyD88 and Wek and Wek binds both MyD88 and dSarm, Wek functions like a hinge downstream of Toll-6 to facilitate signaling via MyD88 or
dSarm, resulting in alternative outcomes. Remarkably, adaptor expression profiles change over time, switching the response to Toll-6 from cell survival to cell death. In the embryo, when both MyD88 and dSarm are abundant, there is virtually no Wek, and Toll-6 can only bind MyD88 to promote cell survival (Fig. 8 C). As Wek levels rise, Toll-6 signaling can also induce cell death. If the Wek-Sarm-JNK route prevails, Toll-6 induces apoptosis; if the Wek–MyD88–NF-κB route prevails, Toll-6 signaling induces cell survival (Fig. 8 C).

Thus, the cellular outcome downstream of DNTs and Tolls is context and time dependent. Whether a cell survives or dies downstream of DNTs and Tolls will depend on which proteases are expressed nearby, which ligand it receives and in which form, which Toll or combination of Tolls it expresses, and which adaptors are available for signaling (Fig. 8).

How adaptor profiles come about or change is not understood. A neuronal type may be born with a specific adaptor gene expression profile, or Toll receptor activation may influence their expression. In fact, MyD88 reinforces its own signaling pathway, as Toll-6 and -7 up-regulate Dorsal, Dif, and Cactus protein levels (McIlroy et al., 2013) and TLR activation increases Sarm levels (O’Neill and Bowie, 2007). We showed that apoptosis caused by MyD88 excess depends on JNK signaling. Because JNK functions downstream of Wek and dSarm, this suggests that MyD88, presumably via NF-κB, can activate the expression of JNK, wek, or dsarm. By positively regulating wek expression, MyD88 and dSarm could establish positive feedback loops reinforcing their alternative pathways (Fig. 8 C, bottom). Because dSarm inhibits MyD88, mutual regulation between them could drive negative feedback. Positive and negative feedback loops underlie pattern formation and structural homeostasis and could regulate neuronal number in the CNS as well. Whether cell-autonomous or -nonautonomous mechanisms result in the diversification of adaptor profiles, either in time or cell type, remains to be investigated.

Either way, over time the Toll adaptors segregate to distinct neural circuits, where they exert further functions in the CNS (Fig. 8 C). Toll-1, -6, and -8 regulate synaptogenesis in distinct neural circuits, where they exert further functions in the motor system (McLaughlin et al., 2016).

Materials and methods

Genetics

Mutant and reporter stocks. Control stocks were yw and/or outcrosses of yw, as most transgenic flies were in a w- background. MyD88<sup>1a6</sup> is an ethyl methanesulfonate (EMS)-induced hypomorphic allele (a gift from B. Mousian; Charatsi et al., 2003), and wek<sup>761C</sup> is an excision loss-of-function allele (a gift from J.L. Imler, Centre National de la Recherche Scientifique, Strasbourg, France; Chen et al., 2006). dsarm<sup>107</sup> and dsarm<sup>261</sup> are loss-of-function alleles of dsarm (a gift from Marc Freeman, University of Massachusetts Medical School, Worcester, MA). The deficiency Df(2R)BSC279 lacks the MyD88 locus and Df(2L)BSC690 lacks the wek locus, respectively. Dorsal-GFP (w<sup>118</sup>; P{Bac(dl-GFP,FLAG)VK00033/TM3, Stb<sup>1</sup>) and Dif-GFP (w<sup>118</sup>; Phac[Dif-GFP,FPTB]VK00033) are both GFP exon trap lines, and Ect4<sup>00080</sup> and sarm are synonyms for the same gene, and Ect4<sup>Pombe-GFP</sup> (yw; MIMicEct4[M108854]) is a MIMIC insertion bearing GFP into the Ect4 locus. Stocks were balanced using CyO;lacZ and TM6BlacZ, to identify mutant embryos, or SM6aTM6B balancers carrying Tb<sup>-</sup>, to identify mutant larvae and pupae. Double and triple mutants and other stocks were generated by conventional genetics.

Overexpression in vivo. We used the following GAL4 drivers: (a) w;; elav-GAL4 for all neurons; (b) w; GMR-GAL4 for the retina (a gift from Matthew Freeman, University of Oxford, Oxford, England, UK); (c) w; RG-GAL4 drives expression in the ring gland and in a small neuronal cluster in the optic lobes; and (d) w; MyD88-GAL4: yw; [P{GawB}[MyD88<sup>886094</sup>/CyO, P{UAS-lacZ.UW14}/UW14] (Bloomington Drosophila Stock Center). These were crossed to (a) the membrane-tethered reporter w;; 10xUAS-myr-tid-Tomato (a gift from B.D. Pfeiffer, University of Texas Southwestern, Dallas, TX); (b) activated forms of Tolls w;; UAS-Toll-6<sup>IC</sup> and w;; UAS-Toll-7<sup>IC</sup> (McIlroy et al., 2013) and UASToll-1<sup>10b</sup> (a gift from J.M. Reichhart, University of Strasbourg, Strasbourg, France); (c) w; UAS-MyD88-FL (a gift from J. Kagan, Harvard Medical School, Boston, MA); (d) w; UAS-dsarm (a gift from Marc Freeman), which drives expression of the dsarm cDNA (Osterloh et al., 2012), and w<sup>118</sup>; P{EP}[EP]3610/TM6B, Tb<sup>-</sup>, which drives expression of all Ect4 (dsarm) isofoms (Bloomington Drosophila Stock Center; Ect4 and sarm are synonyms for the same gene); (e) UAS-wek-HA; UAS-cactus-HA (FlyORF); or (f) w<sup>111</sup>; UAS-JNK-RNAi [P{GD10555}] (VDRC34138) and UAS-dsarm-RNAi; UAS-MyD88-RNAi (VDRC32396; Vienna Drosophila Research Center).

Structural modeling of DNTs and comparison to mammalian NTs

DNT1 and 2 were modeled on their closest structural homologue, Spz, which drives expression of all potential isoforms (Bloomington Stock Center). These were crossed to (a) w;; P{GawB}[Spz<sup>886094</sup>/CyO, P{UAS-MIMicEct4[M108854]}) (a MIMIC insertion bearing GFP into the Ect4 locus. Stocks were balanced using CyO;lacZ and TM6BlacZ, to identify mutant embryos, or SM6aTM6B balancers carrying Tb<sup>-</sup>, to identify mutant larvae and pupae. Double and triple mutants and other stocks were generated by conventional genetics.

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the loops that were not observed crystallographically in Spz. The same method was used to complete the 3D model of Spz. The structure of the BDNF protomer is known in the context of heterodimerization with either NT3 (Robinson et al., 1995) or NT4 (Robinson et al., 1999). We generated a 3D model of the BDNF homodimer based on these heterodimers by substituting the NT with BDNF and performing energy minimization in Modeller (Webb and Sali, 2014). Protein sequences were analyzed by Clustal Omega (Sievers et al., 2011) and Tcoffee (Notredame et al., 2000). Figures were generated in PyMol (DeLano Scientific) using the lowest energy models with least clashes and best geometry according to Verify3D (Bowie et al., 1991) and MolProbity (Chen et al., 2010), respectively.

Bioinformatics and sequence analysis

Analysis of prodomain. Analysis was performed using PSIPRED, a secondary structure prediction program.

Identification of conserved furin sites. Potential furin cleavage sites in DNT1 and 2 were identified by the PiTou prediction tool (Agilent Technologies). For truncated, or mutant forms of DNT1 or 2 cloned into constructs were generated by site-directed mutagenesis (see the Site-directed mutagenesis section). S2 cells were transfected with full-length, truncated, or mutant forms of DNT1 or 2 cloned into pAct5c-3×HA expression vector (see the Cell culture, transfection, stimulation . . . section). After transfection, cells were separated from culture media and lysed in NP-40 buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, and 1% Igepal CA-630). HA-tagged proteins in cell lysates and culture media were detected by anti-HA antibody using standard Western blots.

Primer design. Primers were designed using the public resource Primer3Plus. For site-directed mutagenesis, primers were designed using the QuickChange Primer Design online tool (Agilent Technologies). For qRT-PCR to detect which Toll receptors were expressed in S2 cells, Primer-BLAST was used to design specific primers.

Molecular biology

Generation of fusion constructs. Full-length or truncated cDNAs of DNT1 and 2 were cloned into an expression vector using a standard Gateway procedure, inserting them first into pDONR and subsequently into pAct5c-3×HA to generate the following constructs: pAct5c-DNT1-FL-3×HA; pAct5c-DNT1 (Sp + CK + CTD) – 3×HA; pAct5c-DNT2-FL-3×HA; and pAct5c-DNT2 (Sp + CK) – 3×HA. Cloning to generate HA-tagged dsarm was also performed using the Gateway system. dsarm cDNA was amplified from a pUAST-dsarm plasmid (a gift from Marc Freeman) and then was subcloned first into pDONR and subsequently into the destination vector, resulting in pAct5c-dsarm-3×HA. UAS-DNT1-FL-GFP and UAS-DNT2-FL-GFP were tagged at the C terminus with GFP by cloning: DNT1-FL-GFP was cloned into pUASl using conventional ligation and transgenesis, and DNT2-FL-GFP was cloned by Gateway cloning into pUAS-GW-GFP followed by conventional transgenesis, using white as the selection marker. For all primers, see Table S1.

Generation of MyD88cr2.8 mutant allele by CRISPR/Cas9. A MyD88 CRISPR mutant allele was created by designing a guide RNA targeting exon 1 of MyD88 using CRISPR software (Massachusetts Institute of Technology) with the primers MyD88 BbsI sense, 5′-GTCGCCGGAGGGATTATGGACCTC-3′, and antisense, 5′-AAA CGAGATCCATACCTCCGG-3′, cloned in to the BbsI site of the pCFD3 U6.3 vector and verified by sequencing. Transgenic flies bearing U6.3 MyD88 guide RNA were generated by qC31 transgenesis (injections by BestGene Inc). Flies bearing the guide RNA (yssc: U6.3MyD88gRNA attp2/TM3(sb)) were crossed to flies carrying Cas9 driven by the nanos promoter ymlnosCas9/ZH2A. Independent balanced stocks were established from F1 males (w;MyD88CRISPR/CYO) and sequenced. MyD88cr2.8 bears a 7-bp deletion that causes a frame-shift at amino acid 64 and a premature stop codon at amino acid 94. This corresponds to the start of the death domain (amino acids 90–172). This allele lacks the death and Toll–interleukin receptor domains and is therefore a null allele. The sequence of the lesion and the amino acid sequence are given in Table 1.

qRT-PCR. qRT-PCR was performed to see which Toll receptors were expressed in S2 cells. Total RNA was isolated from S2 cells by TRizol (Ambion) reagent following a standard protocol. Reverse transcription was performed by using the GoScript system (Promega). The standard PCR reaction was performed to amplify Toll receptor cDNA fragments using Taq DNA polymerase (Invitrogen). For a list of primers, see Table S1.

Site-directed mutagenesis. One or more point mutations were generated in pAct5c-DNT1-FL-3×HA and pAct5c-DNT2-FL-3×HA fusion constructs by site-directed mutagenesis according to Wang and Malcolm (1999). The following mutant expression clones were used for S2 cell transfection: pAct5c-DNT1-FL-R499G-3×HA, pAct5c-DNT1-FL-R283/499G-3×HA, pAct5c-DNT2-FL-R284G, and pAct5c-DNT2-FL-R214/221/284G-3×HA. For primers, see Table S1.

qRT-PCR. From 2 h–staged egg collections at 25°C, whole dechorionated embryos were harvested 20 h after egg laying (AEL), and the CNS was dissected from L2 larvae at 48 h AEL, L3 larvae at 96 h AEL, and pupae 0–12 h after pupariation formation. Samples were then placed immediately into TRI reagent (AM9738; Ambion) and frozen at −80°C. Total RNA was extracted from 20 embryos or 20 dissected larval or pupal CNSs using TRI and following the manufacturer's instructions. cDNA was synthesized from 200 ng of total RNA using the GoScript reverse transcription system (A5001; Promega) using random primers and then diluted threefold for quantitative PCR reactions.

Table 1. Lesion and amino acid sequences used to generate the MyD88cr2.8 allele

<table>
<thead>
<tr>
<th>Allele</th>
<th>Sequence (5′–3′)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MyD88WT</td>
<td>GTCAGTATCCGCGCTTATCCGACCGCTGCGACGTGCGCCACAGAGTATGCACTC</td>
</tr>
<tr>
<td>MyD88cr2.8</td>
<td>GTCAGTATCCGCGCTTATCCGACCGCTGCGACGTGCGCCACAGAGTATGCACTC</td>
</tr>
<tr>
<td>MyD88cr2.8</td>
<td>GGTCCGGATGTCACCAGCGAAGCGCGCTACCTGCAACCAGCTTACATCCGCA</td>
</tr>
<tr>
<td>MyD88cr2.8</td>
<td>GCTCCGCATCAGCCGAAAGCGCTTGGCCACGCTTACCAAGCACTTACATCCGCA</td>
</tr>
<tr>
<td>MyD88cr2.8</td>
<td>MRPRFVCQHSHYQPHSHHHTHHHHPNPHHHYHTYGTDVSYRVRRTAGVMVAE</td>
</tr>
<tr>
<td>MyD88cr2.8</td>
<td>MRPRFVCQHSHYQPHSHHHTHHHHPNPHHHYHTYGTDVSYRVRRTAGVMVAE</td>
</tr>
<tr>
<td>MyD88cr2.8</td>
<td>GVMDSGCGSGTGTGL----G-HFNETPLSALIGETRTQLSRMLRKKVLRSEEGYRDW</td>
</tr>
<tr>
<td>MyD88cr2.8</td>
<td>GVMQGRDRARERAWGSTPRHPWASRPAPSCPCASTOP</td>
</tr>
</tbody>
</table>

Guide RNA sequences are given in bold.
and 2 µl was used per reaction. “No reverse transcription” controls were run alongside cDNA reactions. Transcript levels were determined in triplicate for each sample using SensiFAST Hi-ROX SYBR green (BIO-92020; Bioline) run on a sequence detection system (ABI PRI SM 7000; Thermo Fisher Scientific). The reference gene was RpL32, as it remained constant over the course of development. Primers used are given in Table S1.

To obtain fold change values by using the 2^{-ΔΔCt} method (Livak and Schmittgen, 2001) for the developmental profiles of MyD88, dsarm, and wek, the Ct value of RpL32 was subtracted from the Ct value of each gene and developmental time point to obtain ΔCt. All values were then normalized to the calibrator, which, for this set of experiments, was MyD88 mRNA at embryo (ΔΔCt). Three independent biological replicates were performed per experiment, and the mean ± SD is provided in Fig. 5 B.

Cell culture

Cell culture, transfection, stimulation, and subcellular fractionation. S2 cells were maintained at 27°C in InsectXpress medium (Lonza) supplemented with 10% heat-inactivated FBS and 1% penicillin–streptomycin–glutamine (Gibco). Transfection reagent (TransIT2020; Mirus) was used to express target proteins in S2 cells.

To stimulate S2 cells with mature DNT2-CK, S2 cells were transfected with pAct5c-Toll-6-3×HA or pAct5c-Toll-7-3×HA and were grown overnight in a 6-well plate (2 × 10^6 cells/well). Cells were serum starved for at least 6 h and then were treated with purified DNT2-CK (50 nM) for 5–60 min.

To separate nuclear and cytoplasmic fractions, cells were pelleted and washed in ice-cold PBS at 500 g for 5 min at 4°C. The cells were lysed in ice-cold harvest buffer (10 mM Hepes, pH 7.9, 50 mM NaCl, 0.5 M sucrose, 0.1 mM EDTA, 0.5% TritonX-100, 1 mM DTT supplemented with a protease inhibitor cocktail [Thermo Fisher Scientific] and 5 mM NaF, and 2 mM Na_3VO_4) for 5 min on ice. Lysate was spun at 800 g for 10 min at 4°C. Supernatant was treated as cytoplasmic/membrane and pellet was treated as nuclear fraction. The cytoplasmic/membrane fraction was transferred in an empty tube and subsequently purified by centrifugation at 14,000 g for 10 min at 4°C. The nuclear pellet was resuspended in buffer A (10 mM Hepes, pH 7.9, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM DTT supplemented with protease inhibitor cocktail and 5 mM NaF, and 2 mM Na_3VO_4) and spun at 800 g for 10 min at 4°C. Supernatant was discarded and the pellet was resuspended in buffer C (10 mM Hepes, pH 7.9, 500 mM NaCl, 0.1 mM EDTA, 0.1 mM EGTA, 0.1% NP-40, 1 mM DTT supplemented with a protease inhibitor cocktail and 5 mM NaF, and 2 mM Na_3VO_4) and incubated on ice for 30 min. Nuclear fraction was purified by centrifugation at 14,000 g for 10 min at 4°C.

To analyze total cell lysate, S2 cells were pelleted and washed in ice-cold PBS and then lysed in radioimmunoprecipitation assay buffer (10 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.1% SDS, 0.1% sodium deoxycholate supplemented with a protease inhibitor cocktail and 5 mM NaF, and 2 mM Na_3VO_4). Total cell lysate or subcellular fractions were analyzed by SDS-PAGE followed by Western blotting using standard procedures.

Coimmunoprecipitations from cotransfected S2 cells. Coimmunoprecipitations from S2 cells were performed as previously described (McIroy et al., 2013). S2 cells were transfected with the following combinations of plasmids: (a) pAct5c-Toll-6-3×HA and pAct5c-MyD88-V5; (b) pAct5c-Toll-7-3×HA and pAct5c-Wek-3×HA; (c) pAct5c-dsarm-3×HA and pAct5c-Toll-7-3×Flag; or pAct5c-Toll-7-3×Flag and pAct5c-dsarm-3×Flag. pAct5c-MyD88-V5 plasmid was a gift from S. Wasserman (University of San Diego, San Diego, CA); pAct5c-Wek-3×HA was a gift from J.L. Imler (Institut de Biologie Moléculaire et Cellulaire, Strasbourg, France). Cells were collected 48 h after transfection and lysed in NP-40 buffer or in Flag affinity chromatography buffer (50 mM Hepes, pH 7.5, 80 mM KCl, 5 mM MgCl_2, 2 mM EGTA, and 0.2% Triton X-100) supplemented with protease inhibitor cocktail. Immunoprecipitations from lysates were performed using mouse anti-V5 antibody in combination with protein-A/G magnetic beads (Thermo Fisher Scientific) or anti-Flag antibody-conjugated agarose or magnetic beads (Sigma-Aldrich). Proteins were analyzed by SDS-PAGE and Western blotting as described in the Western blots section.

Luciferase reporter assay in mammalian cells. HEK293 cells were seeded at 10^5 cells/well in a 96-well plate 3 h before transfection with jetPEI (Polyplus). NF-κB–dependent gene expression was determined using a luciferase reporter construct concomitantly with indicated TLR vectors. The Renilla luciferase-thymidine kinase–encoding plasmid (pRL-TK) was used to normalize for transfection efficiency, and pcDNA3.1 empty vector was used to maintain constant DNA.

Cells were stimulated in a dose-dependent manner using neurotrophic agents hNGF-β (H9666; Sigma-Aldrich), hBDNF (R&D Systems), or mNGF-7S (N0513; Sigma-Aldrich). Transfected cells were lysed using Passive lysis buffer and assayed for luciferase and Renilla activity using luciferase assay reagent (Promega). Luminescence readings were corrected for Renilla activity and expressed as fold increases over unstimulated control values. Data are presented as means ± SEM of one of three independent experiments. Statistical analysis was performed using a two-way analysis of variance where we compared TLR signaling upon stimulation with varying concentrations of NTs or upon stimulation with both canonical innate immunity ligands and NTs.

Immunostainings

In vivo immunostainings in the larval and pupal CNS. Dissections, fixations, and immunostainings were performed following standard procedures, except that for stainings to detect apoptosis in the pupal CNS, only pupae within the first 10 min of puparium formation were used in order to minimize biological variability in apoptosis levels over time. Primary antibodies used were rabbit anti-GFP (1:500 in larvae and pupae, 1:1,000 in embryos; A11122; Invitrogen), rabbit anti-DesRed (1:100; 632496; Takara Bio Inc.), rabbit anti-pJNK (1:5,000; Cappel), mouse anti-Eve (1:5–1:10; 2B8; Developmental Studies Hybridoma Bank), mouse anti-Eve (1:20; 3C10; Developmental Studies Hybridoma Bank), mouse anti-pERK (1:500 in retina and 1:100 in optic lobe; 9106; Cell Signaling Technology), mouse anti-Repo (1:250; 8D12; Developmental Studies Hybridoma Bank), rabbit anti-pJNK (1:200; V7931; Promega), rabbit anti-MiyD88 (1:250; a gift from S. Wasserman), and rabbit anti-Dcp1 (cleaved Drosophila Dcp1 [Asp216]; 1:500; 95788; Cell Signaling Technology). Secondary antibodies were directly conjugated Alexa Fluor 488, 546, and 647 (1:250, Molecular Probes) or biotinylated mouse or rabbit secondary antibodies (1:2,000; Vector Laboratories) or the Tyramide Signal Amplification kit (T20922; Thermo Fisher Scientific), using the manufacturer’s instructions. For sample sizes, see Table S2.

Western blots. Western blotting was performed according to standard procedures. Primary antibodies were used: mouse anti-V5 (1:5,000; R960-25; Invitrogen), rabbit anti-Flag (1:2,000; F7425; Sigma-Aldrich), mouse anti–histone-H1 (1:10,000; 05-629; EMD Millipore), mouse antitubulin (1:10,000; T9026; Sigma-Aldrich), chicken anti-FA (1:2,000 and 1:5,000; E-7140; Aves Lab), mouse anti-HA (12CA5; 1:200; 11 583 816 001; Roche), mouse anti-Dorsal (7A4; 1:500; Developmental Studies Hybridoma Bank), mouse anti-
Cactus (3H1; 1:500; Developmental Studies Hybridoma Bank), and rabbit anti-DRP (1:500; a gift from D. Ferrandon, University of Strasbourg, France). Secondary antibodies used were anti-mouse HRP (1:5,000; PI-2000; Vector Laboratories), anti-rabbit HRP (1:5,000; PI-1000; Vector Laboratories), and anti-chicken HRP (1:10,000; 703-035-155; Jackson ImmunoResearch Laboratories, Inc.).

Microscopy and imaging

Imaging. For microscopy, samples were mounted either in 70% glycerol and 30% PBTriton (larval and pupal fluorescent CNS and non-fluorescent embryos) or in Vectashield (H-1000; Vector Laboratories; fluorescent embryonic CNS). Wide-field microscopy was performed with a microscope (Axioskop 2; ZEISS) and a 63× objective; images were taken under Nomarski optics with an AxioCam color camera and Zen software (ZEISS). Fluorescent microscopy was performed using secondary antibodies directly conjugated to Alexa Fluor 488, 546, and 647. Laser-scanning confocal microscopy was performed at room temperature using a spectral confocal microscope (SP2 AOBSS; Leica Microsystems) and a 40× or 63× lens at 512 × 512 pixels resolution and with 1 µm steps or a laser-scanning confocal microscope (LSM 710; ZEISS) with a 25× oil lens at 512 × 512-pixel resolution and with 1-µm steps. Confocal image acquisition was performed with Leica Microsystems or ZEISS software as per the system. Each confocal stack comprised 100–300 images, which were processed as follows: (a) for image data, ImageJ (National Institutes of Health) was used to view the entire stack of images, carry out horizontal and transverse projections, and rotate images; occasionally, a median or “dust and scratches” filter was applied to a projection image over the whole image. Photoshop (Adobe) was used to adjust levels, rotate and crop images, and adjust image size to 300 dpi. Illustrator (Adobe) was used to compile figure plates. (b) For quantitative data (e.g., number of Dcp1+, Eve+, or YFP+ cells), we used the ImageJ plugins DeadEasy Larval Glia (which counts nuclear stains) and DeadEasy Caspase for Larvae (for apoptotic cells) as previously described and validated (Forero et al., 2009, 2012; Kato et al., 2011). DeadEasy analyzes the entire stacks of images in 3D, identifies cells based on pixel intensity and 3D volume, and counts cells automatically in an entire CNS in 3D in less than a minute.

Quantitative data analysis. Penetration is the frequency with which a phenotype is manifested within a population, and expressivity is the severity of the phenotype. Eve+ cells in embryos analyzed under wide-field Nomarski optics were counted manually under an Axioskop 2 microscope and a 63× objective. Fluorescent pJNK+ cells in the retina were counted manually within the stacks of confocal sections using ImageJ and the Cell Counter macro.

Dcp1+ apoptotic cells from the entire VNC of the CNS were counted automatically using DeadEasy Caspase for Larvae (Forero et al., 2009), specific for apoptotic cells and optimized for the larval/pupal CNS (Kato et al., 2011). The entire VNC was counted, using the edges of the optic lobes as anterior boundaries. Eve+ cells in the larval CNS were counted automatically with DeadEasy Larval Glia software, which counts nuclear stains (see previous section; Forero et al., 2012). For Eve+ cell counting, the thoracic (T1–T3) and posterior tip cells were excluded because cells there are too packed together, and only the cells from abdominal segments A1–A6 were counted.

Quantification of pixel intensity was performed with ImageJ, setting fixed regions of interest over the area posterior to the morphogenetic furrow or over the morphogenetic furrow, and the mean intensity in this area was normalized over the mean intensity of a fixed region of interest over the eye disk anterior to the morphogenetic furrow.

Statistical analysis. Statistical analyses were performed using SPSS (IBM) and Prism (GraphPad Software). Continuous data (e.g., the number of Dcp1+, pJNK+, and Eve+ cells) were analyzed first for normality, using curve shape or kurtosis and skewness, and then testing the homogeneity of variance with a Levene’s test. If the Levene’s test was not significant, a one-way analysis of variance was used, and Welch analysis of variance was used if samples did not pass the Levene’s test. Multiple genotypes were compared with a single control with Dunnett’s post-hoc test or were compared with each other using Bonferroni’s multiple comparison corrections tests. For TLR signaling, data were analyzed using a two-way analysis of variance, and Dunnett’s post-hoc tests were used for multiple comparisons to NT = 0 controls. For genetic experiments, reproducibility was confirmed by the overall large population sizes and consistent results in multiple repetitions of the experiments; for cell culture data, qRT-PCR, and coimmunoprecipitations, the experiments were performed at least three times. All p-values, tests, and sample sizes are provided in figure legends, and further details are in Table S2.

Online supplemental material

Fig. S1 shows how structural analysis of the prodomains of Spz, DNT1, and DNT2 revealed unique features in each ligand. Fig. S2 shows S2 cells expressing Toll-I, -2, -5, -7, -8, and -9. Fig. S3 shows how DNT1 and 2 bind Toll-6 and -7 promiscuously. Fig. S4 shows the penetrance of Eve+ neuron number phenotypes in the embryonic CNS. Fig. S5 shows how mammalian NTs elicit signaling from TLR4 and alter the response of several TLRs to their canonical immunity ligands. Table S1 is a list of primers used, and Table S2 is a list of all genotypes, sample sizes, and statistical analysis details.

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References


Carvalho, L., A. Jacinto, and N. Matova. 2014. The Toll/NF-
innate immunity: an


caspase: automatic counting of apoptotic cells in Drosophila.

DeLotto, Y., and R. DeLotto. 1998. Proteolytic processing of the
Spätzle protein by easter generates a dimeric NGF-like molecule with


Mizuguchi, P., and P. Schierhorn. 2008b. Crystallization of Spätzle, a cysteine-knot protein involved in embryonic


Gong, Y., P. Cao, H.J. Yu, and T. Jiang. 2008. Crystal structure of the neurotro-


Hoffmann, A., P. Neumann, A. Schierhorn, and M.T. Stubbbs. 2008b. Crystallization of Spätzle, a cysteine-knot protein involved in embryonic
Figure S1. Structural analysis of the prodomains of Spz, DNT1, and DNT2 reveals unique features in each ligand. Pink barrels indicate α-helices, orange arrows indicate β-strands, lines indicate coils, and sequences in yellow highlights in DNT1 and 2 indicate the putative sequences that might correspond to the Spz α-helix involved in the activation of Toll-1 (yellow and boxed). This helix is absent in DNT1 and 2, suggesting that their mechanism for receptor activation differs from that of Spz and Toll-1.
Figure S2. **S2 cells express Toll-1, -2, -5, -7, and -8.** (A) Western blots showing the purity of subcellular fractionation samples for Fig. 3 C; antitubulin is restricted to the cytoplasmic/membrane (C/M) fraction, and anti–histone-1 is enriched in the nuclear fraction (N). Molecular masses are given in kilodaltons. (B) RT-PCR from S2 cells using primers to each of the Tolls showing the presence of bands corresponding with expected product sizes, which are indicated on the left. The experiment was repeated in two consecutive passages of S2 cells, shown here as top and bottom gels. GAPDH was used as a house-keeping control. Negative control reactions were performed in the absence of reverse transcription. S2 cells express Toll-1, -2, -5, -7, and -8, but not Toll-6.

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Figure S3. **DNT1 and 2 bind Toll-6 and -7 promiscuously.** Coimmunoprecipitations from S2 cells cotransfected with Toll-6–HA or Toll-7–HA and DNT1-CK-CTD-V5 or DNT2-CK-V5, respectively. The first three panels are controls. On the right, precipitating the ligands with anti-V5 brought down the receptors detected in Western blots with anti-HA. IP, immunoprecipitation; mock, no transfection; WB, Western blot. Molecular masses are given in kilodaltons.
Figure S4. Penetrance of Eve+ neuron number phenotypes in the embryonic CNS. (A–E) Percentage bar charts showing phenotypic penetrance of decreases in Eve+ neuron numbers. (A) Dorsal anterior corner cell (aCC), pCC, and RP2 Eve+ neurons are lost most prominently in MyD88 Toll-7 Toll-6 triple mutants. $\chi^2$: P < 0.0001, Bonferroni’s multiple comparisons correction; ***, P < 0.001. n = 52–170. (B) Loss of dorsal anterior corner cell, pCC, and RP2 Eve+ neurons in dsarm mutants. $\chi^2$: P = 0.0002, Bonferroni’s multiple comparisons correction; ***, P < 0.001. n = 41–170. (C) Loss of Eve lateral (EL) + U/CQ Eve+ neurons in MyD88, Toll-7, Toll-7 loss-of-function, double, and triple mutants. $\chi^2$: P = 0.0002, Bonferroni’s multiple comparisons correction; **, P < 0.01; ***, P < 0.001. n = 28–119. (D) Eve+ Eve laterals and U/CQs are lost in embryos overexpressing sarm in all neurons and in dsarmc705/dsarm4621 mutants. $\chi^2$: P = 0.0002, Fisher’s post-hoc exact test and Bonferroni’s correction; **, P < 0.01; ***, P < 0.001. n = 33–106. (E) Eve laterals and U/CQs are lost in embryos overexpressing wek in all neurons. Fisher’s exact test: ***, P < 0.001. n = 106 and 269. (F and G) Phenotypic penetrance of increases in the number of Eve+ neurons. (F) The number of dorsal Eve+ neurons in the anterior corner cell, pCC, and RP2 increases in dsarm mutant embryos. $\chi^2$: *, P < 0.05. n = 41 and 170. (G) The number of Eve lateral + U/CQ Eve+ neurons increases in dsarm mutant embryos. Fisher’s exact test: **, P < 0.01. n = 16 and 91. Asterisks on graphs refer to post-hoc multiple comparisons corrections. For statistical details, p-values, and sample sizes, see Table S2.
Figure S5. Mammalian NFs elicit signaling from TLR4 and alter the response of several TLRs to their canonical immunity ligands. HEK293T cells were transfected with TLR2, 4, and 5 and stimulated with varying concentrations of purified NGF and BDNF CK dimers, and their canonical ligands activated or modulated a luciferase reporter NF-κB signaling readout. NGF and BDNF induced signaling from TLR4 but not TLR2 or 5. Two-way analysis of variance: for TLR4 BDNF, P < 0.0001; for TLR4 NGF, P < 0.0001. Dunnett’s post-hoc multiple comparison corrections to NT = 0 controls, three repeats per experiment. NGF and BDNF altered the response of TLR4, 2, and 5 to their canonical ligands involved in innate immunity. This suggests either that BDNF and NGF compete with the canonical ligands to activate TLRs, or they bind other receptors that then modify signaling by TLRs. Two-way analysis of variance: P < 0.0001. Dunnett’s post-hoc multiple comparison corrections to NT = 0 controls, three repeats per experiment. Error bars indicate SEM. Asterisks on graphs refer to multiple comparisons corrections: *, P < 0.05; **, P < 0.01; ***, P < 0.001. All data are from three replicates. CNT, control; LPS, lipopolysaccharide. For statistical details and sample sizes, see Table S2.
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Table S2 is a separate Excel file and contains a list of all genotypes, sample sizes, and statistical analysis details.